

8th February, 1966

I.T.Co.(of G.B.& I.)PROGRAMME

Bioassay studies by long-term skin painting of I.C.I./S.P.F. mice. In progress at Huntingdon Research Centre. Started August 1965.

Under test:

Condensates from EMBASSY cigarette rods fitted with:

- (a) Acetate filters
- (b) Dual filters - 10 mm. Myria (T section)/  
5 mm. Acetate (M section)
- (c) Dual filters - 10 mm. Myria + 9% PEI  
(T section)/ 5mm, Acetate  
(M section)
- (d) Dual filters - as (c), but smoked into the  
filter

I.T.Co. are also testing:

- (e) Condensate from EMBASSY rods to which PEI has  
been added at the rate of 0.1 mg./cigarette.  
This is to assess the possible effects of PEI  
transfer to the smoke.

It is understood that, for each experiment, groups of 250 mice are being painted with doses of 25, 50 or 100 mg. condensate.

B-A.T. - BATTELLE PROGRAMME

Ciliary Inhibition Tests under Project CONQUEROR - prefixed with C. Skin painting tests (short- or long-term) under Project JANUS - prefixed with J.

105610868

Project CONQUEROR

Test CA1: Measurement of ciliastatic activity of cigarette smoke using clam gill preparations, based on the test devised by Celanese Corporation of America. Clam gill tissue is exposed to successive puffs (20 ml.) of cigarette smoke and the ciliary beat frequency is measured stroboscopically in the inter puff period. Results expressed as the percentage inhibition of ciliary beat on a puff-by-puff basis and plotted graphically. Area under curve measured (P) and used as one measure of ciliastatic activity.

Ten tissue preparations tested per sample and results expressed as a graph of number of preparations killed against number of puffs taken. A second measure of ciliastatic activity is the number of puffs (T) which would kill all tissue samples, i.e., 100% ciliastatic activity.

A third measure is the relative dose effect (ED<sub>50</sub>), expressed as the ratio of the number of puffs from a sample required to kill 50% of the preparations to the corresponding number from the control cigarette.

An activity rating (BZ<sub>A1</sub> = Bewertungsziffer) can be calculated.

$$BZ_{A1} = \frac{P}{5} + 5 \cdot ED_{50} + T$$

3

Test CA2: Measurement of the inhibition of particle transport using preparations of the rabbit trachea in vitro, based on the technique published by Kensler and Battista. Rabbit trachea preparations are exposed to successive puffs (25 ml.) of cigarette smoke and the particle transport rate measured in the inter puff period. Results expressed as percentage decrease in rate as a function of numbers of puffs and plotted graphically. Area under curve (P) measured.

Ten preparations tested per sample and the other two measures (T and ED<sub>50</sub>) derived.

Activity Rating BZ<sub>A2</sub> calculated as above.

Results from Tests CA1 and CA2 are then combined for each cigarette sample.

105610869

$$BZ = \frac{BZ_{A1} + BZ_{A2}}{2}$$

The lower the value of BZ, the more toxic is the smoke sample.

Test CB: Goblet cell proliferation in rat lung.

Rats are exposed in a chamber to the smoke from a selected cigarette sample over a period of weeks, with a daily exposure of up to 8-10 cigarettes. An equal number of control animals are exposed to air only - dummy smoking. Animals are removed at intervals from both the control and experimental groups, sacrificed and sections of trachea and bronchi made and stained with Alcian/PAS to reveal mucous-secreting (goblet) cells. The number of goblet cells per high power field are counted. Up to 20 high power fields per specimen are examined and the results averaged.

The technique is not proving easy and we await a report from Battelle before deciding whether it is worth pursuing further.

Project JANUS

Tests JA1 and JA2: These are based on the Harrogate Short Term Hyperplasia test (see T.R.C. programme, item 2.2).

Under test JA1, four cigarette samples are examined per month. For each sample, a group of 48 mice is taken and six are sacrificed at the start of the experiment to act as controls. The remainder are painted daily with doses of 100 mg. fresh smoke condensate for three or five days, and after each painting a group of six mice, chosen at random, is sacrificed. Thus the schedule is:-

Day 1	Sacrifice 6 mice.	Paint 42 mice.
Day 2	Sacrifice 6 mice.	Paint 36 mice.
Day 3	Sacrifice 6 mice.	Paint 30 mice.
Day 4	Sacrifice 6 mice.	(Paint 24 mice.)
Day 5	Sacrifice 6 mice.	(Paint 18 mice.)
Day 6	Sacrifice 6 mice.	
Day 7	Sacrifice 6 mice.	
Day 8	Sacrifice <u>6</u> mice.	
	Total	<u>48</u> mice

Tissue is taken from the painted area of the sacrificed mice, fixed, mounted and stained (haematoxylin-eosin). The thickness of the epithelium is measured at several points and the thickness plotted graphically against time in days.

105610870

Whereas in theory Test JA1 was to be devoted to the routine examination of cigarette samples, Test JA2 was to have been a research investigation into the methodology of the hyperplasia test in order to optimise the procedure. In fact, the results at Battelle have differed from what was understood to be the results from Harrogate in several important directions.

- (a) Hyperplasia does not reach a maximum on the day following the last painting.
- (b) Thickening does not occur logarithmically.
- (c) Extensive necrosis is observed with fresh smoke condensate.

Consequently, both Tests JA1 and JA2 have latterly been devoted to an examination of methodology in order to attempt to reproduce the results understood to have been obtained at Harrogate, and it appears clear that the hair cycle of the mouse is a factor to be considered.

Test JB: Long Term skin painting.

Facilities for carrying out long-term skin painting tests are being established at Battelle. It is planned to test a total of 10 samples of smoke condensate. Each test will be carried out on a total number of 750 S.P.F. mice derived from the I.C.I. strain, split into three groups of 250 mice each on one of three dose levels or subdivided in some other way, e.g., two equal groups tested with different condensates at one dose level. The aim will be to use a sufficiently large number of animals to permit detections of a difference in specific carcinogenicity of, say, 25-50%, with reliable statistical accuracy.

A group of 250 mice as controls - 125 untreated and 125 painted with solvent only - will be added to the first experiment and further groups may be needed later in the series of tests in order to check the base line.

Tests will be started at three-monthly intervals. Each time a batch of mice is received, a further group (50 or 100 mice) will be treated with doses of standard carcinogens (benzpyrene, dibenzanthracene) in order to calibrate their response and to ensure that there has been no large change in susceptibility.

In the first instance, mice will be painted with solutions of freshly assayed (24-hour old) smoke condensate. Later, it may be possible to use "instant" condensate. Provision has been made, therefore, for all condensate

105610871

production to be made at Battelle.

Originally, it had been planned to start long term skin painting studies under Test JB on April 4th 1966, but there have been delays in the construction of the buildings, which will entail some postponement of the start.

105610872

T.R.C. BIO-ASSAY PROGRAMME

1. ASSESSMENT OF MOUSE SKIN CARCINOGENESIS ON NON-VOLATILE SMOKE CONDENSATE OF U.K. CIGARETTES

1.1. Testing T<sub>1</sub> Cigarettes (Composite Flue-cured Blend). Started 1.5.63.

1.1.1. 24 hour old condensate at 25, 50 and 100mg, three times a week with 660 mice per dose.

Total mice - 1980.

1.1.2. One-month old condensate; doses, frequency and numbers of mice as in 1.1.1.

1.1.3. Neutral Fraction at doses equivalent to 25, 50 and 100mg of non-volatile whole smoke condensate (= approx. 10, 20 and 40 mg neutral fraction).

Total mice - 1980.

1.1.4. Neutral Fraction (large dose) - started 8.11.63.

Doses of 200 and 300 mg of neutral fraction used.

Total mice - 200.

1.1.5. Neutral Fraction and Hydrocarbon Fractions - started 9.12.63.

Neutral - 100 mg - 100 mice.

Hydrocarbon Fraction - 133 mg - 100 mice.

Neutral Fraction <sup>minus</sup> hydrocarbons - 133 mg - 100 mice.

Recombined - 67 mg - 100 mice.

} See Fig. 1.

1.2. Testing Water Soluble and Insoluble Fractions of Cigarette Smoke Condensate from T<sub>1</sub> Cigarettes (Experiment started 18.11.63)

1.2.1. Ether extract of water soluble fraction (Fraction E) - 100 mice.

1.2.2. Water insoluble fraction - 100 mice.

1.2.3. Re-combined 1.2.1. and 1.2.2. - 100 mice.

1056108/5

1.3. Effect of Nicotine as a Possible Anti-Carcinogen

1.3.1. Addition to Benzpyrene (experiment started 3.11.63)

- 1.3.1.1-4. 3:4 Benzpyrene alone and with 2.5, 5.0 and 10 mg of nicotine tartarate.  
3:4 Benzpyrene concentration - 0.3 ml of a 0.0063% solution in acetone/30% water.  
150 mice per test.  
Total mice - 600.

1.3.2. Addition to Neutral Fraction (experiment started 17.2.65)

- 1.3.2.1. Neutral Fraction + Tartaric acid - 100 mg - 100 mice.  
1.3.2.2. " " + 2.5 mg nicotine tartarate - 100 mg - 100 mice.  
1.3.2.3. " " + 5.0 mg nicotine tartarate - 100 mg - 100 mice.  
1.3.2.4. " " + 10.0 mg nicotine tartarate - 100 mg - 100 mice.

1.3.3. Addition to Hydrocarbon Fraction (experiment started 17.2.65)

- 1.3.3.1. Hydrocarbon fraction + Tartaric acid - 100 mg - 100 mice.  
1.3.3.2. " " + 2.5 Nicotine Tartarate - 100 mg - 100 mice.  
1.3.3.3. " " + 5.0 mg Nicotine Tartarate - 100 mg - 100 mice.  
1.3.3.4. " " + 10.0 mg Nicotine Tartarate - 100 mg - 100 mice.

1.4. Testing T<sub>4</sub> Cigarette and Improved Fractionation Scheme

The T<sub>4</sub> Cigarette is comparable to the T<sub>1</sub> Cigarette, but is of later manufacture. The improved fractionation scheme is shown separately on Figure 2, and has been applied to condensate from both T<sub>1</sub> and T<sub>4</sub> cigarettes.

105610874

T<sub>1</sub> Cigarette Fractionation (experiment started 5.8.64)

- 1.4.1. T<sub>1</sub> cigarette - Fraction D - ether insoluble, water soluble  
fraction - 160 mg - 400 mice.
- 1.4.2. " " Fraction E - ether soluble, water soluble  
fraction - 160 mg - 390 mice.
- 1.4.3. " " Fraction F - methanol fraction -  
160 mg - 390 mice.
- 1.4.4. " " Fraction G - cyclohexane fraction -  
160 mg - 390 mice.
- 1.4.5. " " Fractions D, E, F & G recombined -  
40 mg - 390 mice.

T<sub>4</sub> Cigarette (experiment started 2.11.64)

- 1.4.6. T<sub>4</sub> cigarette - non-volatile whole smoke condensate -  
100 mg - 240 mice.
- 1.4.7. 24 hour condensate from T<sub>4</sub> cigarette -  
25 mg - 234 mice  
50 mg - 234 mice  
100 mg - 234 mice

T<sub>4</sub> Cigarette Fractionation (experiment started 10.2.65)

- 1.4.8. T<sub>4</sub> cigarette - Fraction B (water soluble) - 100 mg - 300 mice.
- 1.4.9. " " Fraction C (water insoluble) - 100 mg - 300 mice.
- 1.4.10. " " Recombined B + C - 100 mg - 300 mice.
- 1.4.11. " " Fraction D, + E + C - 100 mg - 300 mice.  
(experiment 1.4.11. started 3.5.65).

1.5. Testing of Different Cigarettes

- 1.5.1. T<sub>5</sub> cigarette - Low tar/normal nicotine (I.T.Co. blend).  
24 hour old condensate at 25, 50 and 100 mg doses;  
each dose using 234 mice.  
(Experiment started 2.11.64.)

105610875

- 1.5.2. T<sub>12</sub> - tobacco treated with copper nitrate - 100 mg - 100 mice.
- 1.5.3. T<sub>11</sub> - " " " copper potassium malate - 100 mg - 100 mice.
- 1.5.4. T<sub>7</sub> - " " " malic acid - 100 mg - 100 mice.
- 1.5.5. T<sub>10</sub> - " " " potassium nitrate - 100 mg - 100 mice.
- 1.5.6. T<sub>8</sub> - " " " potassium malate - 100 mg - 100 mice.
- 1.5.7. T<sub>9</sub> - " " " potassium hydrogen malate -  
100 mg - 100 mice.

(Experiments 1.5.2. - 1.5.7. started 3.5.65.)

- 1.5.8. T<sub>6</sub> } 25 mg - 100 mice.
- 1.5.9. T<sub>6</sub> } Control tobacco for T<sub>7-13</sub> 50 mg - 100 mice.
- 1.5.10. T<sub>6</sub> } 100 mg - 100 mice.
- 1.5.11. T<sub>13</sub> } Tobacco treated with 25 mg - 100 mice.
- 1.5.12. T<sub>13</sub> } Potassium Carbonate 50 mg - 100 mice.
- 1.5.13. T<sub>13</sub> } 100 mg - 100 mice.

(Experiments 1.5.8. - 1.5.13. started 26.5.65.)

1.6. Ad Hoc. Experiments

- 1.6.1. T<sub>4</sub> cigarettes - non-volatile whole smoke condensate -  
100 mg - 150 mice.
- 1.6.2. T<sub>4</sub> cigarette + cellulose acetate filter - 75 mg - 150 mice.
- 1.6.3. As in 1.6.2. with polyethylene glycol on the filter -  
75 mg - 100 mice.
- 1.6.4. T<sub>4</sub> cigarette + VPA Charcoal filter (Cigarette Components) -  
75 mg - 150 mice.
- 1.6.5. Comparison of non-volatile whole smoke condensate from German  
homogenised tobacco and German cigarettes.  
Folie B - 75 mg - 100 mice.  
Blend B - 75 mg - 100 mice.

1.7. Experiments About to Start

- 1.7.1. Composite Cigar ,
  - 1.7.2. Cigar Tobacco in Cigarette Form (T<sub>4</sub> specification) } at 3 dose levels,  
12.5, 25 and  
50 mg.
  - 1.7.3. T<sub>4</sub> Cigarettes as control.
- Total number of mice - around 1000.

10001000

1.7.4. T<sub>4</sub> Cigarettes - Fraction H at 2 levels - 740 mice.

There has since been some alteration in the frequency of dosing used in the experiments already under way at Harrogate. An examination of the effects of different frequencies has been built in to experiments 1.7.1. to 1.7.3., and will continue to be a part of all future experiments until completed.

It is also intended to examine, as soon as possible, cigarettes made from Burley, air-cured fermented and oriental tobaccos. It is also hoped to examine T<sub>4</sub> cigarettes to which have been attached filters containing P.E.I.

1.8. T.R.C. Experiments Carried Out Elsewhere

1.8.1. Comparison of flue-cured and air-cured tobacco (T<sub>2</sub> and T<sub>3</sub>).

Skin painting tests carried out by Dr. F.J.C. Roe at the Institute of Cancer Research Laboratories at Pollard Wood.

1.8.1.1. 3 groups of 400 mice painted with the old non-volatile whole smoke condensate from the flue-cured cigarettes (T<sub>2</sub>) and the air-cured cigarettes (T<sub>3</sub>). Doses were 60 mg given 3 times per week.

1.8.1.2. 3 groups of 100 mice for co-carcinogenicity comparison. Paintings with

	dimethylbenzanthracene + T <sub>2</sub> condensate
"	+ T <sub>3</sub> "
"	alone

1.8.2. Comparison of the non-volatile whole smoke condensate from T<sub>1</sub> and U.S.A. non-filtered King Size cigarettes. The experiment is being carried out by Dr. E. Wynder in New York.

1.8.3. Comparison of cigarettes made from flue-cured, cigar and Burley (yet to be done) tobaccos. This work is being carried out by Professor R.D. Passy at the Chester Beatty Research Institute.

1.8.4. Comparison of the specific carcinogenic activity of non-volatile whole smoke condensate from T<sub>4</sub> with and without MISO. This work is being carried out at the Huntingdon Research Centre.

1056108/1

2. DEVELOPMENT OF OTHER BIO-ASSAY TECHNIQUES

2.1. A smoking machine has been produced at the Battelle Institute, Frankfurt, which will produce condensate around 30 seconds old. This machine is now being evaluated at Harrogate.

2.2. Immediate Response of Mouse Skin to Condensate (Hyperplasia)

In this test, condensate is painted on the back of a mouse every 24 hours. A total number of 3 paintings per mouse are done, and a number of mice are sacrificed after every painting. A specimen of skin is then removed and the increase in thickness measured. It was hoped that this test would be useful as a screening procedure for the long term skin painting experiments.

2.3. Tests Designed to Produce Epidermoid Carcinoma

2.3.1. Intra-Pulmonary Injection of Condensate (Blacklock).

- 2.3.1.1. Condensate plus vehicle - 100 rats.
- 2.3.1.2. Benzpyrene plus vehicle - 100 rats.
- 2.3.1.3. Vehicle only - 100 rats.
- 2.3.1.4. Control - 100 rats.

Animals will be sacrificed serially at 3-monthly intervals.

2.3.2. Experiments to find Easier Techniques

- 2.3.2.1. Intratracheal injection - discontinued.
- 2.3.2.2. Intrapleural injection - discontinued.

2.3.3. Pulmonary Installation of Condensate (Shabad)

- 2.3.3.1. Condensate (3 doses) plus carbon - 300 S.P.F. rats.
- 2.3.3.2. Benzpyrene plus carbon - 100 S.P.F. rats.
- 2.3.3.3. Carbon only - 100 S.P.F. rats.
- 2.3.3.4. Control - 100 S.P.F. rats.

Animals will be sacrificed at the rate of approximately 10/month in each test.

1056108

2.4. Tissue Culture

Work is under way at Harrogate in an attempt to reproduce and use the technique of Lasnitzki involving foetal tissue.

2.5. Inhalation Tests to Produce Carcinoma

2.5.1. Standard lung carcinogens applied as an aerosol.

2.5.2. Inhalation of smoke by mice.

2.5.2.1. Leutchenberger technique using intermittent puffing (discontinued).

2.5.2.2. Harris Technique. Two smoking runs per day or ten per week using Harris-type inhalation chamber and S.P.F. C57 Black mice. Mice will be sacrificed in groups of 20 at monthly intervals. 600 mice required for the experiment.

2.5.2.3. Further exploratory experiments.

(a) Inhalation of smoke and carbon aerosol primer.

(b) Use of mice previously infected with influenza virus.

(c) Development of smoke administration machine.

2.6. Other Bio-assay Tests

Tests of smoke on lung clearance function such as cilia and mucous flow.

2.6.1. Goldhamer technique with kittens - discontinued.

2.6.2. Dalhamn in vivo technique - discontinued.

2.6.3. In vitro work with fresh water clams. The method was established at the Huntingdon Research Centre and is now being transferred to Harrogate.

2.6.4. Tests involving the measurement of ciliary beat frequency using mammalian tissue from the rabbit. A test has been developed by the Pharmacology unit and is now being transferred to the bio-assay section for routine use.

105610879

3. T.R.C. WORK BEING UNDERTAKEN OUTSIDE THE HARBOR GATE LABORATORIES

3.1. The testing of T<sub>2</sub> and T<sub>3</sub> cigarettes by the Imperial Cancer Research Fund. Dr. Harris is exposing mice to the smoke of T<sub>2</sub> and T<sub>3</sub> cigarettes, the mice having been previously infected with influenza virus.

Four groups of mice are being treated as follows:-

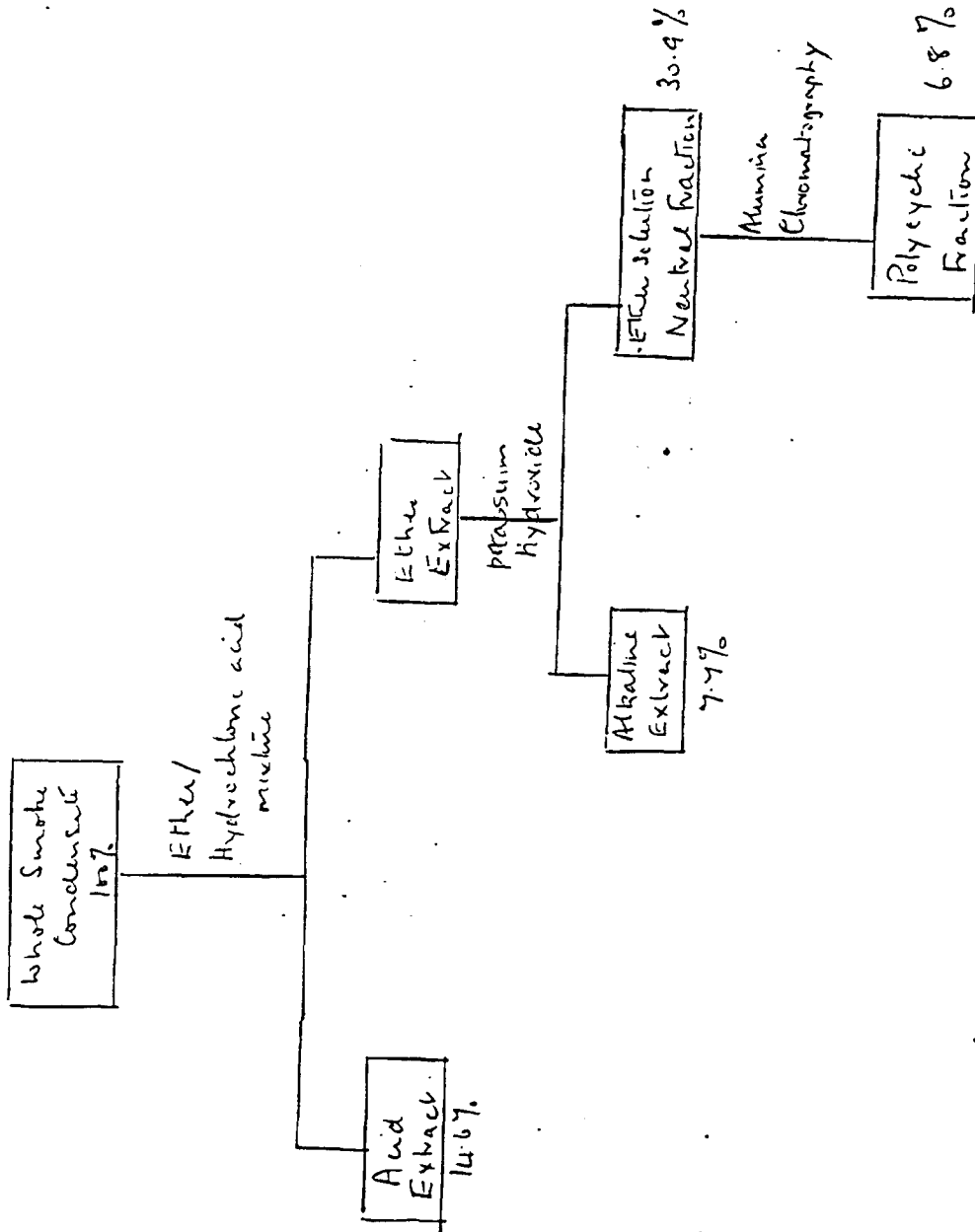
- (a) Virus infection alone.
- (b) Virus infection followed by daily exposure to aerosol of 3:4 benzpyrene.
- (c) Virus infection followed by daily exposure to T<sub>2</sub> and T<sub>3</sub> smoke.
- (d) Virus infection followed by daily exposure to aerosol of benzpyrene and T<sub>2</sub> and T<sub>3</sub> smoke.

3.2. Development of Inhalation Techniques at Battelle.

3.3. Feasibility Study on the Ability of Primates to Smoke and Inhale.

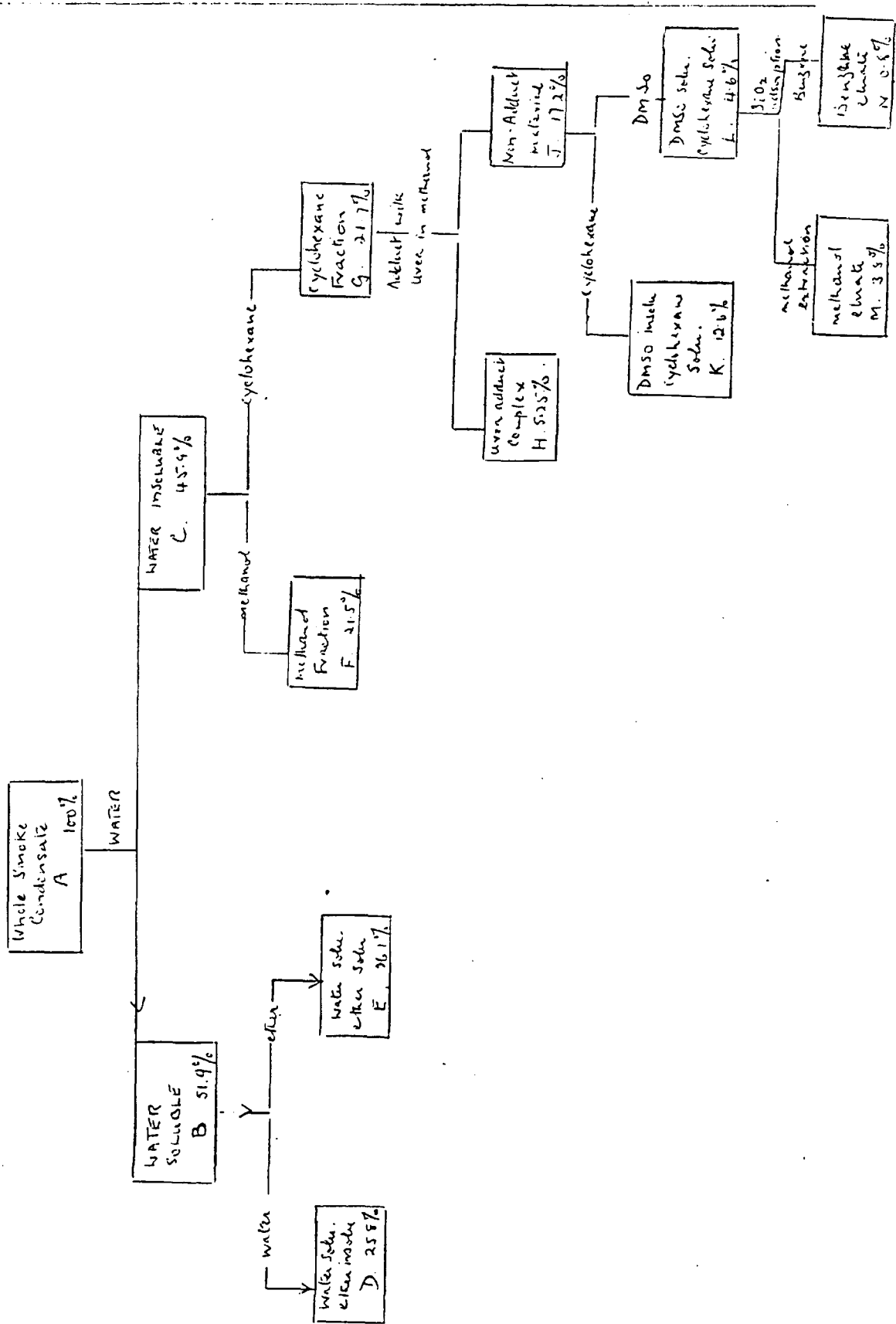
105610980

Fig I  
Original Fraction Scheme



105610881

Fig 2  
Improved Fractionation Scheme



105610882